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The Immunosuppressant FK506 and Its Nonimmunosuppressive Analog L-685,818 Are Toxic to *Cryptococcus neoformans* by Inhibition of a Common Target Protein

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The immunosuppressant FK506 (tacrolimus) is an antifungal natural product macrolide that suppresses the immune system by blocking T-cell activation. In complex with the intracellular protein FKBP12, FK506 inhibits calcineurin, a Ca^{2+}-calmodulin-dependent serine-threonine protein phosphatase. We recently reported that growth of the opportunistic fungal pathogen *Cryptococcus neoformans* is resistant to FK506 at 24°C but sensitive at 37°C and that calcineurin, the target of FKBP12-FK506, is required for growth at 37°C *in vitro* and pathogenicity *in vivo*. These findings identify calcineurin as a potential antifungal drug target. In previous studies the calcineurin inhibitor cyclosporin A (CsA) was effective against murine pulmonary infections but exacerbated cryptococcal meningitis in rabbits and mice, likely because CsA does not cross the blood-brain barrier. Although we find that FK506 penetrates the CNS, FK506 also exacerbates cryptococcal meningitis in rabbits. Thus, FK506 immunosuppression outweighs antifungal action *in vivo*. Like FK506, the nonimmunosuppressive FK506 analog L-685,818 is toxic to *C. neoformans in vitro* at 37°C but not at 24°C, and FK506-resistant mutants are resistant to L-685,818, indicating a similar mechanism of action. Fluconazole-resistant *C. neoformans* clinical isolates were also found to be susceptible to both FK506 and L-685,818. Our findings identify calcineurin as a novel antifungal drug target and suggest the nonimmunosuppressive FK506 analog L-685,818 or other congeners warrant further consideration as antifungal drugs for *C. neoformans*.

Cryptococcus neoformans is an opportunistic fungal pathogen that primarily infects immunocompromised individuals. Infection initiates in the lung following inhalation, spreads hematogenously to the brain, and then infects the meninges to cause severe, often fatal, meningoencephalitis (10, 28, 33). *C. neoformans* infection is one of the most common opportunistic infections in AIDS patients, particularly in areas without widespread use of fluconazole. The inadequacies of present treatment regimes for cryptococcal meningitis are shown by the fact that AIDS patients require lifelong suppressive therapy with fluconazole to prevent relapse. A new worrisome development is the appearance of fluconazole-resistant strains (5, 42). Finally, other antifungal agents used to treat cryptococcal infections, such as amphotericin B, have serious toxic side effects. Therefore, the identification of additional antifungal drugs, and of potential drug targets, is of paramount importance for improving management of invasive mycoses such as cryptococcosis.

The immunosuppressants cyclosporin A (CsA), FK506 (tacrolimus), and rapamycin (sirolimus) are natural products with antimicrobial activities, and it has been suggested that these compounds might have clinical applicability as novel antibiotics. FK506 is toxic to the pathogenic fungus *Aspergillus fumigatus* (20), to *Neurospora crassa* (1), and to unusual mutant strains of *Saccharomyces cerevisiae* (4, 7, 16, 25, 41, 49). We recently reported that FK506 is toxic to the opportunistic fungal pathogen *C. neoformans* at 37°C but not at 24°C *in vitro* (39a), suggesting that the target of FK506 might be required for pathogenicity in mammalian hosts, whose body temperature is equal to or greater than 37°C. If so, FK506, or its derivatives, might have anticryptococcal activity *in vitro* and *in vivo*.

FK506 suppresses the immune system by inhibiting signal transduction events required for T-cell activation in response to antigen presentation to the T-cell receptor (reviewed in references [8, 22, and 45]). FK506 binds to a family of intracellular binding proteins, the FKBP immunophilins (19, 47). FKBPs are enzymes that catalyze *cis-trans* peptidyl-prolyl isomerization. FK506 binding potently inhibits this enzymatic activity but does not account for drug action (3, 11, 22). Instead, the FKBP12-FK506 complex binds to and inhibits a Ca^{2+}-calmodulin dependent serine-threonine specific protein phosphatase, calcineurin (30, 31). In T cells responding to antigen presentation, a rise in intracellular Ca^{2+} activates calcineurin, which subsequently dephosphorylates the NF-AT transcription factor, allowing nuclear import and expression of genes encoding interleukin 2 (IL-2) and other T-cell activation factors (9, 14, 26, 39, 40, 46).

Previous studies in the yeast *Saccharomyces cerevisiae* reveal that the mechanisms of immunosuppressive and antifungal action of FK506 are remarkably similar (4, 7, 15, 22, 24, 25, 37). FK506 diffuses into yeast cells and binds the immunophilin FKBP12. The yeast and human FKBP12 proteins share 54% identity (24, 48) and have virtually superimposable structures, as revealed by X-ray crystallography (44, 51). The FKBP12-FK506 complex then inhibits yeast calcineurin, resulting in an inability to recover from pheromone-induced cell cycle arrest, sensitivity to cation stress and, in some mutant strains, inviability (4, 7, 15, 16, 25, 37, 41, 49).

In previous studies, another immunosuppressive drug that inhibits calcineurin, cyclosporin A (CsA), was found to reduce cryptococcal pulmonary infections in mice (34). However, CsA

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exacerbated cryptococcal meningitis in both mice and rabbits, most likely because CsA does not effectively penetrate the central nervous system (CNS) (35, 43). Although previous studies suggested that FK506 can cross the blood-brain barrier, which we have confirmed in this study, we find that FK506 also exacerbates cryptococcal meningitis in immunocompromised rabbits. These observations suggest that the immunosuppressive effects of FK506 overcome any potential antifungal activity *in vivo*. To circumvent this, we have identified a nonimmunosuppressive FK506 analog, L-685,818 (18-OH, 21-ethyl-FK506) (2, 12), which retains antifungal activity *in vitro*. Like FK506, L-685,818 was toxic at 37°C but not at 24°C. In addition, *C. neoformans* mutants selected for FK506 resistance were also resistant to L-685,818. Taken together, these findings indicate FK506 and L-685,818 share a similar mechanism of action in yeast but not in mammalian cells. Fluconazole-resistant *C. neoformans* clinical isolates are susceptible to both FK506 and L-685,818. Our findings provide evidence that an FKBP12-FK506 complex is toxic in *C. neoformans* via inhibition of calcineurin and suggest further studies of the nonimmunosuppressive FK506 analog L-685,818, or other nonimmunosuppressive analogs, as novel antifungal agents are warranted.

MATERIALS AND METHODS

Strains, growth inhibition assay, and compounds. The pathogenic serotype A strain H99 has been described (50). Strains JEC20 and JEC21 are isogenic MAT**a** and MATα derivatives of serotype D strain B3501 (36) and were provided by J. Edman (University of California, San Francisco). Spontaneous FK506-resistant mutants from strains JEC20 and JEC21 (C20F1, C20F2, C21F2, and C21F3) are as described elsewhere (39a). Strains T1 and 89-610 are fluconazole-resistant clinical isolates kindly provided by Mahmoud Ghannoum (UCLA-Harbor) and John Graybill (San Antonio, Tex.). FK506 was from Fujisawa, L-685,818 and FK520 from Merck, and rapamycin from NCI. Stock solutions were in dimethyl sulfoxide (DMSO) at 10 mg/ml and stored at −20°C.

In vitro susceptibility tests of *C. neoformans* strains, and determinations of MIC and minimum fungicidal concentration (MFC) were performed by the National Committee for Clinical Laboratory Standards (NCCLS) broth macrodilution method (38). The fungal growth inhibition assay was performed at 0.01-, 0.1-, 1-, and 10-μg/ml drug concentrations. Drug dilutions and inoculum preparation were by the NCCLS criteria (38). Optical density (OD) was read with a Beckman spectrophotometer at 600 nm following incubation for 72 h at 24, 30, and 37°C.

Preparation of protein extracts and ^{3}H-FK506 LH-20 binding assays. Protein extracts were prepared from 100 OD_{600} units of cell pellet by glass bead homogenization in 1 ml of lysis buffer (150 mM Tris [pH 7.5], 10 mM $MgCl_2$, 1 mM dithiothreitol [DTT], 10% [vol/vol] glycerol, 1 mM phenylmethyl sulfonyl [PMSF], 3 μg fluoride of benzamide per 1 μg/ml of aprotinin per ml, and 1 μg of leupeptin per ml) with 5 × 45 s bursts with cooling on ice. Extracts were microcentrifuged at 12,000 rpm for 8 min at 4°C, and supernatants were transferred to fresh tubes. Protein content was quantified by the Bradford method, and extracts were stored at −80°C.

LH-20 assays were as described elsewhere (21) except the carrier protein fetal calf serum was purified bovine serum albumin (BSA) because calf serum contains a ^{3}H-FK506 binding activity ([48a]; unpublished observations). His6-tagged *S. cerevisiae* FKBP12 was overexpressed and purified as described elsewhere (6). ^{3}H-FK506 was custom tritiated at NEN/Dupont by reductive tritiation of the C21 allyl bond (specific activity, 119.8 Ci/mmol).

Rabbit model of cryptococcal meningitis. New Zealand White rabbits weighting 2 to 3 kg were housed in separate cages and provided water ad libitum and Purina rabbit chow. *C. neoformans* serotype A strain H99 was prepared by growth for 96 h at 30°C on Sabouraud's agar medium containing 100 μg of chloramphenicol per ml and resuspended in 0.015 M phosphate-buffered saline (PBS). Six rabbits were administered cortisone acetate at 2.5 mg/kg intramuscularly 1 day prior to inoculation with *C. neoformans* and continuing daily for 14 days. Twenty-four hours following initiation of steroid treatment, rabbits were anesthetized with xylazine and ketamine intramuscularly and inoculated intracisternally with ~10^7 yeasts in a 0.3 ml suspension through a 25-gauge needle on a 3-ml syringe. FK506 was administered at a 1-mg/day dose in the four experimental animals and was started on day 4 and then continued for 10 days. Rabbits were sedated on days 4, 7, 12, and 14 after inoculation, and cerebrospinal fluid (CSF) was withdrawn. Quantitative yeast cultures were by plating serial dilutions of CSF in PBS on YPD medium, incubation at 30°C for 72 h, and counting CFU. The maximum volume of CSF assayed was 0.1 ml, and thus the detection limit of the assay is 10 organisms per ml.

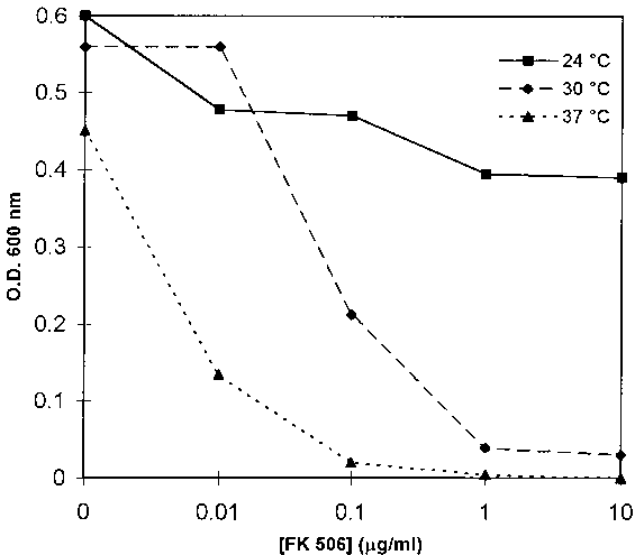

FIG. 1. FK506 is toxic to *C. neoformans* at 37°C but not at 24°C *in vitro*. *C. neoformans* strain H99 was cultured in RPMI medium with the indicated concentrations of FK506 for 72 h at 24, 30, and 37°C. Growth was assayed by determining the optical density at 600 nm. Results presented are representative of three independent experiments.

RESULTS

FK506 is toxic to *C. neoformans*. We recently found the immunosuppressants CsA and FK506 are toxic to *C. neoformans* at 37°C but not at 24°C *in vitro* (39a). In these studies, CsA and FK506 toxicities were determined on solid YPD medium. We extended these findings using the NCCLS growth inhibition criteria for antifungal activity (38). In this assay, *C. neoformans* cells were cultured in liquid RPMI medium for 72 h with increasing FK506 concentrations and growth was assessed by optical density. Representative growth inhibition curves for the pathogenic *C. neoformans* serotype A strain H99 are presented in Fig. 1. FK506 toxicity was readily detectable in this *in vitro* assay, with a MIC of ~0.01 μg/ml at 37°C and ~0.1 μg/ml at 30°C and similar MFC (Table 1). In contrast, little or no growth inhibition was observed at 24°C at up to 1,000-fold the MIC at 37°C. These observations confirm that FK506 is toxic to *C. neoformans* at elevated temperature *in vitro*.

***C. neoformans* expresses ^{3}H-FK506 binding activity.** To determine whether FK506 could be toxic in *C. neoformans* via binding to an FKBP protein, extracts were prepared from *C. neoformans* H99 and assayed for FK506-binding protein. We used the LH-20 assay, in which ^{3}H-FK506 is mixed with protein

TABLE 1. MIC and MFC of *C. neoformans* strains or mutants[a]

Strain	FK506		L-685,818		FK520	
	MIC	MFC	MIC	MFC	MIC	MFC
H99	<0.09	<0.09	3.12	3.12	<0.09	<0.09
C20	<0.09	1.56	6.25	12.5	—[b]	—
C21	<0.09	0.19	6.25	12.5	—	—
C20F1	100	100	50	50	—	—
C20F2	50	100	50	50	—	—
C21F2	50	50	100	100	—	—
C21F3	25	50	100	100	—	—

[a] The data are in micrograms per milliliter.
[b] —, not tested.

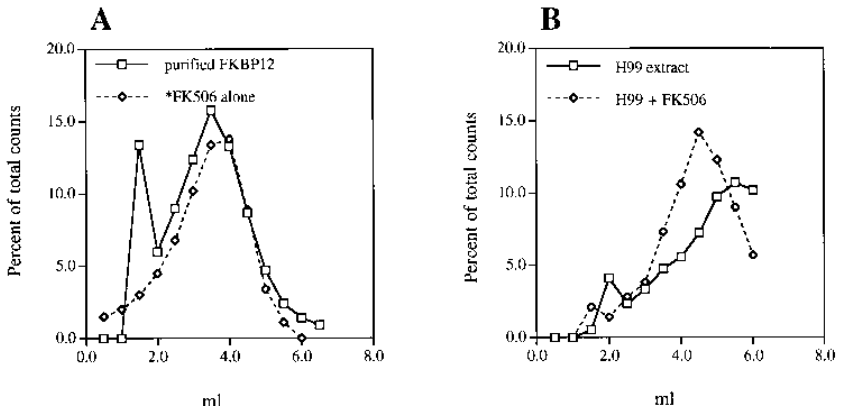

FIG. 2. *C. neoformans* expresses ^{3}H-FK506-binding protein. FK506-binding protein activity was measured by the LH-20 assay. (A) Tritiated FK506 alone or incubated with purified *S. cerevisiae* FKBP12 protein was subjected to LH-20 chromatography, fractions were analyzed by scintillation counting, and the percentages of total counts per minute were plotted. (B) Tritiated FK506 was incubated with protein extract from *C. neoformans* H99 in the absence or presence of excess unlabeled FK506 competitor and subjected to LH-20 chromatography, and the percentages of total counts per minute were plotted.

extract and then chromatographed through Sephadex LH-20, a hydrophobic matrix that retards free FK506 compared to FKBP-FK506 complexes (21). As shown in Fig. 2A, free ^{3}H-FK506 was eluted as a late peak, and ^{3}H-FK506 incubated with purified yeast FKBP12 was eluted in two peaks, an earlier FKBP12-FK506 complex and a later free ^{3}H-FK506 peak. ^{3}H-FK506 incubated with *C. neoformans* protein extract was eluted as an early FKBP-^{3}H-FK506 complex (Fig. 2B). Formation of this FKBP-^{3}H-FK506 was competed by unlabelled FK506 (Fig. 2B) or rapamycin (data not shown). Thus, FK506 toxicity in *C. neoformans* could be mediated by an FKBP-FK506 complex, as in other organisms.

FK506 exacerbates cryptococcal meningitis in rabbits. We next wished to test whether FK506 is toxic to *C. neoformans* in infected animals. Because *C. neoformans* causes meningitis, compounds must cross the blood-brain barrier to have activity. To establish whether FK506 penetrates the CNS, three rabbits (~2.5 kg each) that had been infected with *C. neoformans* meningitis for 7 days were administered a 1-mg intravenous dose of FK506 and drug levels present in whole blood and CSF were determined 30 min later by radioimmunoassay. The mean FK506 levels in whole blood and CSF were 64.7 ± 10.2 and 3.4 ± 1.9 ng/ml (means ± standard errors [SE]), respectively, confirming that FK506 penetrates the CNS at ~5 to 10% of a simultaneous blood level at this time.

We next assessed the *in vivo* effects of FK506 in steroid immunosuppressed rabbits with cryptococcal meningitis. After 3 days of treatment with both FK506 and cortisone, the mean CSF yeast counts ± SEM in the rabbits rose by log 0.54 ± 0.4. On the other hand, rabbits on cortisone alone actually had a drop in yeast counts by log 2.7 ± 0.7. These differences in CSF yeast counts persisted during the 10-day treatment period. Two of the FK506-plus-cortisone-treated rabbits died during therapy on days 3 and 7. FK506 MIC determinations for CSF isolates obtained after treatment confirmed that organisms persisting in one FK506-treated animal retained similar FK506 susceptibility compared to the original isolates.

Nonimmunosuppressive FK506 analog L-685,818 retains antifungal activity *in vitro*. Because FK506 exacerbated *C. neoformans* infections, likely due to further immunosuppression, we tested whether a nonimmunosuppressive FK506 analog L-685,818 retains antifungal activity. In solid YPD growth medium, 1 μg of L-685,818 per ml did not inhibit growth either of *C. neoformans* or of FK506-sensitive *S. cerevisiae* mutant yeast strains (data not shown). Because the toxicity of some compounds is increased in minimal medium and in liquid culture, we tested L-685,818 in the NCCLS growth assay in liquid RPMI media as described above. Under these conditions, L-685,818 inhibited growth of the *C. neoformans* serotype A strain H99 (Fig. 3B; Table 1) and also the B3501-derived serotype D strain JEC20 (Fig. 4A; Table 1). As with FK506, L-685,818 was toxic at 37°C but not at 24°C *in vitro* (Fig. 3B). FK520, an immunosuppressive FK506 analog with the C-21 allyl-to-ethyl substitution of L-685,818 but lacking the C-18 hydroxyl group (17, 30) had similar activity (Fig. 3C). Both FK506 and L-685,818 exhibited similar antifungal activities against two fluconazole-resistant clinical isolates (MICs ≥16 to 32 μg of fluconazole per ml) recently obtained from AIDS patients (strains T1 and 89-610 [data not shown]). Thus, FK506 and the L-685,818 analog are active against *C. neoformans* clinical isolates with decreased azole susceptibility. Our findings extend the earlier *in vitro* studies of L-685,818 and establish that this nonimmunosuppressive FK506 analog has potent antifungal activity.

FK506-resistant *C. neoformans* mutants are resistant to L-685,818. To determine if FK506 and L-685,818 share a similar mechanism of action, we employed FK506-resistant mutants we have recently isolated in the isogenic MATα and MATα serotype D strains JEC20 and JEC21 (39a). Mutant strains C20F1 and C20F2 are isogenic with the wild-type FK506-sensitive strain JEC20, whereas mutants C21F2 and C21F3 derive from JEC21. The mutations segregate as single nuclear mutations which, by heterokaryon analysis, confer recessive drug resistance in three mutants (C20F1, C20F2, and C21F3) and dominant drug resistance in one (C21F2) (39a). Importantly, the three recessive mutations confer resistance to FK506 and to rapamycin, which are both toxic when bound to FKBP12, suggesting that these mutants lack FKBP12.

In the NCCLS growth inhibition assay, both FK506 and L-685,818 inhibited growth of the wild-type JEC20 and JEC21 strains at 37°C (Fig. 4A; Table 1) but not at 24°C (data not shown). In contrast, growth of the isogenic FK506-resistant mutant strains C20F1 (Fig. 4B), C20F2, C21F2, and C21F3 (Table 1) was not inhibited by either FK506 or L-685,818. Thus, FK506-resistant mutants are cross-resistant to L-685,818, indicating a similar mechanism of action which may involve calcineurin inhibition.

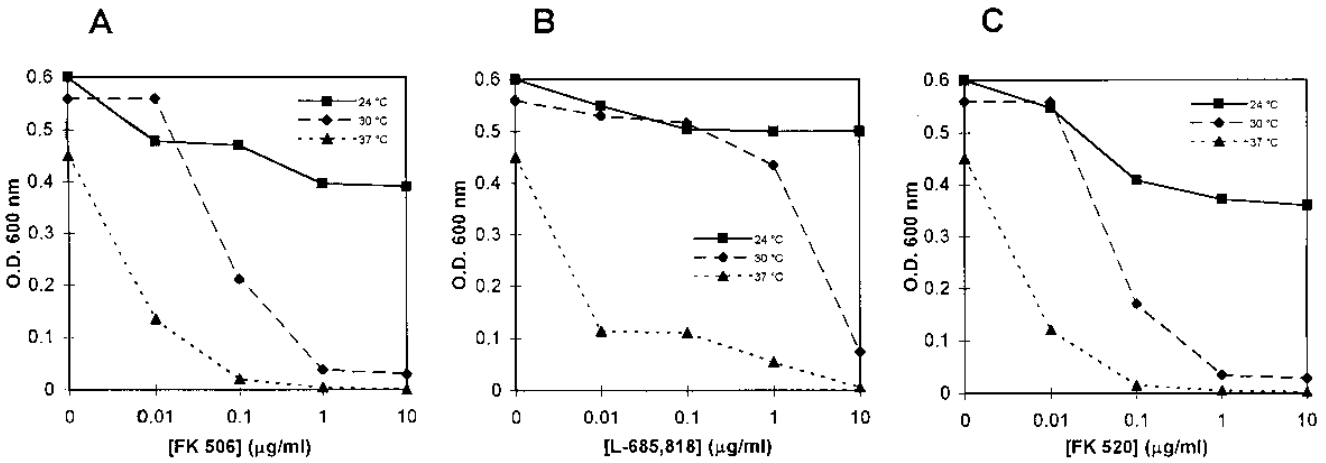

FIG. 3. Nonimmunosuppressive FK506 analog L-685,818 has antifungal activity. *C. neoformans* H99 was cultured in RPMI medium with the indicated concentrations of FK506, L-685,818, or FK520 for 72 h at 24, 30, and 37°C. Growth was assayed by determining the optical density at 600 nm. Results presented are representative of three independent experiments.

DISCUSSION

C. neoformans is an opportunistic fungal pathogen of increasing significance, given that it has a worldwide geographic distribution and is a common infection in AIDS patients and azole-resistant strains are appearing. We recently identified a novel pathogenicity factor in this organism, the protein phosphatase calcineurin, which is required for growth of *C. neoformans* at elevated temperatures and pathogenicity (39a). Calcineurin has come to prominence as the target for the immunosuppressants cyclosporin A and FK506, widely employed in transplant recipients. Our findings suggest that calmodulin, Ca^{2+}, and the pathways that raise intracellular calcium, which are required to activate calcineurin, will similarly be required for virulence of *C. neoformans*. In addition, our findings suggest that there must be one or more specific substrate proteins that are dephosphorylated by calcineurin to allow growth at 37°C *in vitro* and pathogenesis *in vivo*. These findings provide the first outlines of a signal transduction cascade required for fungal pathogenesis and define several novel targets for antifungal drug development.

We began by considering the two known calcineurin inhibitors, cyclosporin A and FK506, as candidate novel antifungal agents. Previous studies demonstrated that CsA exacerbates cryptococcal meningitis in rabbits without directly penetrating into the CNS (43). On the other hand, we demonstrate here that although some FK506 could penetrate into the subarachnoid space, the low drug levels actually appear to exacerbate the CNS infection in a rabbit model of *C. neoformans* meningitis. These findings are supported by the occurrence of cryptococcal infections in human transplant recipients receiving steroids and FK506 (13). However, based on the extent of CNS penetration by FK506 at the dose used in this study, and the *in vitro* MIC for *C. neoformans* grown at 37°C, these doses of FK506 may not result in effective direct antifungal drug in the CNS. Therefore, we cannot exclude the possibility that FK506 confers some antifungal benefit if administered in higher doses. On the other hand, our observations show that the immunosuppressant FK506 exacerbates *C. neoformans* infections *in vivo* even at low doses and support recent clinical reports of transplant recipients receiving FK506 who devel-

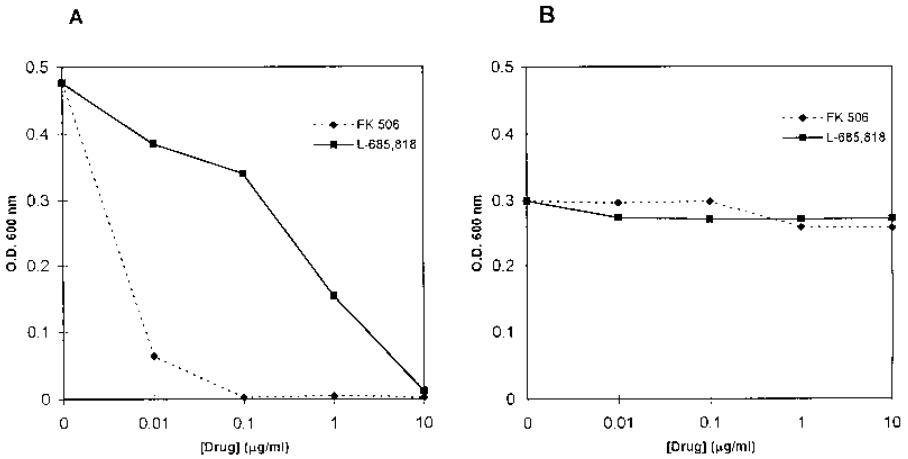

FIG. 4. The FK506-resistant mutant is also resistant to L-685,818. The FK506-sensitive *C. neoformans* JEC20 (A) and the isogenic FK506-resistant mutant strain C20F1 (B) were cultured in RPMI medium with the indicated concentrations of FK506 or L-685,818 for 72 h at 37°C. Growth was assayed by determining optical density at 600 nm. Results presented are representative of three independent experiments.

oped cryptococcosis (13). Thus, neither CsA nor FK506 is itself likely to be clinically useful for treating cryptococcal infections.

We therefore examined nonimmunosuppressive drug analogs with similar fungal targets as candidate antifungal agents. Here we focused on an FK506 analog, L-685,818, which differs from FK506 at two positions on the solvent-exposed effector surface in the FKBP12-FK506 and FKBP12–L-685,818 complexes, which interacts with calcineurin (2, 51, 52). L-685,818 has a hydroxyl group introduced at C-18 that prevents the human FKBP12–L-685,818 complex from binding and inhibiting bovine calcineurin. *In vivo*, L-685,818 is not immunosuppressive and lacks the renal toxicity associated with FK506 (12). Strikingly, L-685,818 antagonizes both the immunosuppressive and the nephrotoxic side effects of FK506 (12), confirming that L-685,818 effectively competes with FK506 for FKBP12 *in vivo* but does not inhibit mammalian calcineurin *in vivo* or *in vitro*. In previous studies, Rotonda et al. reported that, while the human FKBP12–L-685,818 complex does not inhibit bovine calcineurin *in vitro*, the yeast FKBP12–L-685,818 is an effective calcineurin inhibitor (44). Substitutions around the drug binding site may enable yeast FKBP12 to overcome the effect of the introduced C-18 hydroxyl group. These observations suggested that L-685,818 might allow us to take advantage of subtle structural differences between host and fungal FKBP12's, or calcineurins, to inhibit calcineurin-dependent fungal growth but spare T-cell function.

In the NCCLS fungal growth assay, both FK506 and L-685,818 inhibited the growth of several strains of *C. neoformans* that are pathogenic in animals or humans. The minimum fungicidal concentrations (Table 1) show that these compounds are also fungicidal. As with FK506, L-685,818 toxicity was manifested at 37°C but not at 24°C. It is also noted that the L-685,818 antifungal potency has been reduced 10- to 100-fold compared to FK506, but L-685,818 remains a potent anticryptococcal compound. In addition, mutations that confer FK506 resistance also conferred resistance to L-685,818. Taken together, these findings indicate that FK506 and L-685,818 share a similar mechanism of action in *C. neoformans*. Previous studies revealed that single, nuclear, recessive mutations render the C20F1, C20F2, and C21F3 mutant strains resistant both to FK506 and to rapamycin. These observations suggest that these mutants lack the *C. neoformans* homolog of FKBP12 and that an FKBP12-drug complex mediates FK506 and L-685,818 action in this organism. Finally, the findings that L-685,818 is toxic at 37°C but not at 24°C and that calcineurin is required for growth at 37°C but not at 24°C (39a) further support our conclusion that FKBP12–L-685,818 is toxic to *C. neoformans* by inhibition of calcineurin.

Because the nonimmunosuppressive analog L-685,818 retains potent anticryptococcal activity, we propose that the *C. neoformans* FKBP12–L-685,818 complex effectively inhibits *C. neoformans* calcineurin. This activity could result from differences between human and cryptococcal FKBP12, as is the case with *S. cerevisiae* FKBP12 (44). Alternatively, structural differences between mammalian and cryptococcal calcineurins could allow accommodation of the C-18 hydroxy group of L-685,818 by fungal calcineurin. The X-ray structure of the calcineurin-FK506-FKBP12 complex has been solved (18, 27). These structures, and earlier biochemical (29, 32) and genetic studies (7), reveal that FKBP12-FK506 binds in a hydrophobic cleft composed of elements of both the calcineurin A and the calcineurin B subunits. Functional groups on both FK506 and FKBP12 directly contact calcineurin. Sequence differences in FKBP12, calcineurin A, or calcineurin B, could alter FK506 and L-685,818 action. We have isolated the *C. neoformans* gene encoding calcineurin A (39a). All of the calcineurin A residues implicated in FKBP12-FK506 action are conserved from *C. neoformans* to humans. Thus, L-685,818 action is not attributable to calcineurin A changes. *C. neoformans* genes encoding FKBP12 and the calcineurin B subunit have not been isolated but may have changes that account for the toxic effects of L-685,818. These studies may also help to model even more potent L-685,818 antifungal analogs.

Fluconazole-resistant mutants have recently arisen among *C. neoformans* clinical isolates (5, 42). This development is of grave concern given that AIDS patients who have had cryptococcal meningitis require fluconazole prophylaxis for life to prevent relapse. We find that FK506 and L-685,818 are toxic to fluconazole-resistant clinical isolates. It remains to be tested whether L-685,818 confers a beneficial effect in animal models of cryptococcal meningitis with fluconazole-sensitive or resistant mutant strains.

In conclusion, our studies illustrate the value of directed efforts to identify novel targets required for fungal pathogenesis and candidate antifungal agents.

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A.O. and M.D.P. contributed equally to this study.

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